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**(54) SUBSTANCE SPECIFIC TO HUMAN PD-1**

(57) The present invention relates to a substance specific to human PD-1 comprising a part that recognizes human PD-1, a part that recognizes a membrane protein in cell membrane of human PD-1-expressing cells, and linkers. Since the substance specific to human PD-1 selectively can recognize human PD-1 and a mem-

brane protein on cell membrane of human PD-1-expressing cells and can transmit inhibitory signal of human PD-1, it is useful for therapy and/or prevention of diseases caused by immunopathy.

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## Description

## Field of the Invention

- 5 [0001] The present invention relates to antibodies specific to human PD-1, bispecific antibodies comprising antibodies against human T cell receptor complex or human B cell receptor complex, polynucleotides encoding them, and the usage of the bispecific antibodies.

## Background of the Invention

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[0002] The immune system acquired the mechanism that can respond to various foreign antigens. The mechanism takes the diversity of an antigen receptor by recombination of V, (D) and J fragment in T cells and B cells. Although this mechanism brought about a result that produces autoreactive lymphocytes, these autoreactive lymphocytes are removed by the negative selection in thymus or bone marrow, and are further controlled by the self-tolerance mechanism of clonal deletion or anergy in periphery.

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[0003] Although it is thought that an autoimmune disease is developed by the breakdown of self-tolerance, the researches using various disease murine model have been conducted to elucidate the mechanism of pathogenesis. However, the etiology of an autoimmune disease and the molecular mechanism of self-tolerance remain unclear. In such a situation, existence of the mouse which shows the symptoms of an autoimmune disease caused by a single gene deficient is very important for study the etiology of an autoimmune disease from a molecular biological viewpoint. CTLA4-/-mouse which causes lethal systemic lymphocytes infiltration (Waterhouse P., *et al.*, *Science*, 270:985-988, 1995; Tivol E.A., *et al.*, *Immunity*, 3:541-547, 1995), SHP-1 deficient mothaten mice (Shultz L.D., *et al.*, *Cell*, 73:1445-1454, 1993), lyn-/-mouse which shows the symptoms of glomerular nephritis (Hibbs M.L., *et al.*, *Cell*, 83:301-311, 1995), and FCRIIB-/-mouse (Bolland S. & Ravetch J.V., *Immunity*, 13:277-285, 2000) are the representation, and the relations of these molecules and self-tolerance has been studied.

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[0004] The PD-1 gene, which belongs to the immunoglobulin super family, encodes a 55 kDa type I transmembrane protein. Both mouse PD-1 and human PD-1 consist of 288 amino acids, and have signal peptide at N terminal (20 amino acid) and hydrophobic region in the middle part, which is a transmembrane region (*The EMBO J.*, 11(11):3887-3895, 1992; Japanese patent Publication No. 5-336973; EMBUGenBank/DDJB Acc. No. X67914, *Genomics*, 23:704, 1994; Japanese patent Publication No. 7-291996 (U.S. Patent No. 5629204).

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[0005] In thymus, PD-1 is expressed at the transition phase between CD4-/CD8- to CD4+/CD8+ stage on thymocytes (Nishimura H., *et al.*, *Int. Immunol.*, 8:773-780 (1996), Nishimura H., *et al.*, *J. Exp. Med.*, 191:891-898 (2000)). In periphery, PD-1 is expressed on T cells and B cells activated through the antigen receptor (Agata Y., *et al.*, *Int. Immunol.*, 8:765-772 (1996)), and on activated myeloid lineage cells such as macrophages.

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[0006] PD-1 has ITIM (Immunoreceptor tyrosine-based inhibitory motif) in its intracellular region. Therefore, PD-1 is a negative regulator in immune responses. Since PD-1 deficient mice developed a lupus-like glomerular nephritis and arthritis (C57BL/6 genetic background) (Nishimura H., *et al.*, *Int. Immunol.*, 10:1563-1572, 1998, Nishimura H., *et al.*, *Immunity*, 11:141-151, 1999) and dilated cardiomyopathy-like disease (BALB/c genetic background) (Nishimura H., *et al.*, *Science*, 291:319-322 (2001)), it became clear that PD-1 serves as a regulator for the development of autoimmune disease, especially for the maintenance of self tolerance. Further it has been reported that graft rejection is regulated by inhibition of PD-1 signal (*Journal of Immunology*, 169, 11:6543-6553 (2001)),

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## Disclosure of the Invention

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[0007] It is thought that PD-1 is a regulator of various autoimmune diseases, and that it is one of causative genes of autoimmune diseases. Control of the PD-1 function can bring about the medical treatment and diagnosis of suppression or enhancement of the immune function, infection, transplant rejection, and neoplasm *etc.* As a result of keen examination, the present inventors reached the present invention concerning the substances that controls the function of PD-1.

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[0008] Stimuli on lymphocytes which control immunity are transmitted mainly through T cell receptor (TCR) in case of T cells, and B cell receptor (BCR) in case of B cells, and then intracellular phosphorylation plays an important role in the molecular mechanism.

[0009] Since it has been elucidated that PD-1 negatively regulates various immunocompetent cells such as lymphocytes and myeloid cells *etc.* and PD-1 has ITIM (Immunoreceptor tyrosine-based inhibitory motif) in its intracellular region, the present inventors considered that the recruit of de-phosphorylation enzymes (phosphatases) could be involved in the molecular mechanism in the inhibitory signal transduction mediated by PD-1. Therefore, it was hypothesized that making PD-1 colocalize with TCR or BCR can display the PD-1 function.

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[0010] The present inventors confirmed that the inhibitory signal of PD-1 was transmitted with the substance which

physically cross-links PD-1 to TCR or BCR. First, the present inventors confirmed that the above idea was right using anti-PD-1 antibodies and anti-CD3 antibodies. CD3 is a membrane protein expressed on T cells and is one component of TCR complexes. The divalent antibodies were constructed by bridging anti-PD-1 antibodies and anti-CD3 antibodies. The present invention was completed by isolating each cDNA encoding anti-human PD-1 antibody and anti-human TCR antibody and producing bispecific antibody produced by constructing expression vectors that can express fusions proteins comprising each antigen-recognition site of both antibodies.

[0011] The present invention relates to the followings:

- (1) A substance specific to human PD-1 comprising a part that recognizes human PD-1, a part that recognizes a membrane protein on cell membrane of human PD-1-expressing cells, and a linker.
- (2) The substance specific to human PD-1 according to (1), wherein the part that recognizes human PD-1 is an antibody against human PD-1 or a partial fragment thereof.
- (3) The substance specific to human PD-1 according to (1), wherein the part that recognizes a membrane protein on cell membrane of human PD-1-expressing cells is an antibody against the membrane protein or a partial fragment thereof.
- (4) The substance specific to human PD-1 according to (1), comprising an antibody against human PD-1 or a partial fragment thereof, an antibody against the membrane protein on cell membrane of human PD-1-expressing cells or a partial fragment thereof, and a linker.
- (5) The substance specific to human PD-1 according to any one of (1), (3) and (4), wherein the membrane protein is human T cell receptor complex or human B cell receptor complex.
- (6) The substance specific to human PD-1 according to (1) or (4), wherein the linker is a peptide.
- (7) A bispecific antibody comprising an antibody against human PD-1 or a partial fragment thereof, an antibody against human T cell receptor complex or a partial fragment thereof, and a linker.
- (8) A bispecific antibody comprising an antibody against human PD-1 or a partial fragment thereof, an antibody against human B cell receptor complex or a partial fragment thereof, and a linker.
- (9) The bispecific antibody according to (7) or (8), wherein the linker is a peptide.
- (10) A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:2, a homolog thereof, a fragment thereof or a homolog of the fragment, or a polypeptide comprising the amino acid sequence to which 1-10 amino acid(s) of the polypeptide is/are deleted, substituted, and/or added, wherein the polypeptide composes an antibody against human PD-1.
- (11) A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:4, a homolog thereof, a fragment thereof or a homolog of the fragment, or a polypeptide comprising the amino acid sequence to which 1-10 amino acid(s) of the polypeptide is/are deleted, substituted, and/or added, wherein the polypeptide composes an antibody against human PD-1.
- (12) A polypeptide complex comprising the polypeptides according to (10) and (11).
- (13) A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:11, a homolog thereof, a fragment thereof or a homolog of the fragment, or a polypeptide comprising the amino acid sequence to which 1-10 amino acid(s) of the polypeptide is/are deleted, substituted, and/or added.
- (14) A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:11, a homolog thereof, a fragment thereof or a homolog of the fragment, or a polypeptide comprising the amino acid sequence to which 1-10 amino acid(s) of the polypeptide is/are deleted, substituted, and/or added, which is the bispecific antibody according to any one of (7) to (9).
- (15) A polynucleotide encoding the polypeptide according to any one of (10), (11), (13) and (14), a homolog thereof or a complementary polynucleotide thereof, or a fragment thereof or a homolog of the fragment.
- (16) A polynucleotide comprising the nucleotide sequence represented by SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:9, a homolog thereof or a complementary polynucleotide thereof, or a fragment thereof or a homolog of the fragment.
- (17) A duplication or expression vector comprising the polynucleotide according to (15) or (16).
- (18) A host cell transformed by the duplication or expression vector according to (17).
- (19) A manufacturing method of the substance according to any one of (1) to (6), comprising culturing the host cell according to (18) under conditions to express the substance.
- (20) A manufacturing method of the bispecific antibody according to any one of (7) to (9), comprising culturing the host cell according to (18) under conditions to express the bispecific antibody.
- (21) A manufacturing method of the polypeptide according to any one of (10) to (14), comprising culturing the host cell according to (18) under conditions to express the polypeptide.
- (22) A therapeutic and/or preventive pharmaceutical composition for human PD-1 related-diseases, comprising an effective amount of the substance according to any one of (1) to (6), the bispecific antibody according to any one of (7) to (9), the polypeptide complex according to (12), or the polypeptide according to (13) or (14).

(23) The therapeutic and/or preventive pharmaceutical composition according to (22), wherein the human PD-1 related-diseases are diseases selected from neurodegenerative disease, autoimmune disease, collagenosis, organ transplantation rejection, tumour, and infectious disease.

(24) The therapeutic and/or preventive pharmaceutical composition according to (22), wherein the neurodegenerative diseases are diseases selected from geriopsychosis, Alzheimer disease, Down syndrome, Parkinson's disease, Creutzfeldt-jakob disease, diabetic neuropathy, Parkinson syndrome, Huntington's disease, Machado-Joseph disease, amyotrophic lateral sclerosis, diabetic neuropathy, and Creutzfeldt Creutzfeldt-Jakob disease.

(25) The therapeutic and/or preventive pharmaceutical composition according to (22), wherein the autoimmune diseases are diseases selected from glomerular nephritis, arthritis, dilated cardiomyopathy-like disease, ulcerous colitis, Sjogren syndrome, Crohn disease, systemic erythematodes, chronic rheumatoid arthritis, multiple sclerosis, psoriasis, allergic contact dermatitis, polymyositis, pachyderma, periarteritis nodosa, rheumatic fever, vitiligo vulgaris, insulin dependent diabetes mellitus, Behcet disease, Hashimoto disease, Addison disease, dermatomyositis, myasthenia gravis, Reiter syndrome, Graves' disease, anaemia perniciousa, Goodpasture syndrome, sterility disease, chronic active hepatitis, pemphigus, autoimmune thrombopenic purpura, and autoimmune hemolytic anemia.

**[0012]** The part that recognizes human PD-1 in the present invention has only to be a substance that recognizes human PD-1, for example, anti-human PD-1 antibody or a fragment thereof, human PD-1 itself or a fragment thereof, a ligand for human PD-1 or a fragment thereof (Freeman, GJ, *et al.*, *Journal of Experimental Medicine*, 19, 7;1027-1034 (2000)), human PD-L2, and human PD-H3 *etc.*), and a low molecular weight organic compounds *etc.*

**[0013]** The antibody for human PD-1 or a fragment thereof may be either of complete polyclonal or monoclonal antibody, humanized antibody, complete human antibody, and short antibody thereof (for example, F(ab')<sub>2</sub>, Fab', Fab, Fv) *etc.* which contains anti-human PD-1 antibody or a fragment thereof.

**[0014]** Concretely, they are monoclonal anti-human PD-1 antibodies produced by hybridomas (International Trust Number FERM P-19162) that had been deposited to National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary in Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan at Dec 19, 2002, and had been transferred to International Deposit at June 5, 2003 (International Trust Number FERM BP-8392), which was named J110. Though F(ab')<sub>2</sub>, Fab', Fab, and Fv antibody fragments of these antibodies *etc.* are preferable, they don't limit to them.

**[0015]** F(ab')<sub>2</sub>, Fab', Fab, and Fv antibody fragments can be obtained by processing complete antibodies with protease enzyme, and optionally reducing it. They can be produced as antibodies, fragments of thereof, or fusion proteins with another proteins and the fragments, by using the expression vector made by gene rearrangement using the cDNA separated from antibody-producing hybridoma.

**[0016]** The membrane protein on cell membrane of human PD-1-expressing cells means the membrane protein that expresses on cell membrane of the same cell as human PD-1-expressing cells, and includes each the membrane protein of human PD-1-expressing human first stage culture cells or human cell lines. For example, T cell receptor complex or B cell receptor complex is preferable.

**[0017]** The part that recognizes the membrane protein on cell membrane of human PD-1-expressing cells may be a substance that recognizes the membrane protein that expresses on cell membrane of the same cell as human PD-1-expressing cells, and includes, for example, antibodies against the membrane protein and fragments thereof, the membrane protein itself and fragments thereof, ligands for the membrane protein and fragments thereof, and low molecular weight organic compounds that bind to the membrane protein *etc.*

**[0018]** The antibodies or partial fragments thereof to the membrane protein on cell membrane of human PD-1-expressing cells are antibodies or partial fragments thereof to the membrane protein that expresses on cell membrane of the same cell as human PD-1-expressing cells, and may be either of complete polyclonal or monoclonal antibodies, humanized antibodies, complete human antibodies, and short antibodies thereof (for example, F(ab')<sub>2</sub>, Fab', Fab, Fv) *etc.*

**[0019]** T cell receptor complex is at least a complex comprising T cell receptor consisting of  $\alpha$  subunit and  $\beta$  subunit and CD3 consisting of  $\gamma$  subunit,  $\delta$  subunit,  $\epsilon$  subunit, and  $\zeta$  subunit.

**[0020]** B cell receptor complex is at least a complex comprising membrane-bound immunoglobulin, CD79 $\alpha$  subunit, and CD79 $\beta$  subunit.

**[0021]** The antibodies or partial fragments thereof to T cell receptor complex may be either of antibodies or partial fragments thereof that recognize T cell receptor complex. They include antibodies or partial fragments thereof to each subunit of T cell receptor and CD3, which composes T cell receptor complex and may be either of complete polyclonal or monoclonal antibodies, humanized antibodies, complete human antibodies, and short antibodies thereof (for example, F(ab')<sub>2</sub>, Fab', Fab, Fv) *etc.* For example, the monoclonal antibodies against CD3 can be produced by using hybridoma that has already been established. Anti-CD3 ( $\alpha$ -CD3 $\epsilon$ mAb (PharMingen, Inc.)) can be bought.

**[0022]** The antibodies or partial fragments thereof to B cell receptor complex may be either of antibodies or partial fragments thereof that recognize B cell receptor complex. They include antibodies or partial fragments thereof to each

subunit of membrane-bound immunoglobulin and CD79, which composes B cell receptor complex, and may be either of complete polyclonal or monoclonal antibodies, humanized antibodies, complete human antibodies, and short antibodies thereof (for example,  $F(ab')_2$ , Fab', Fab, Fv) etc. Concretely, commercial anti-BCR antibodies can be used and, for example, anti-IgG (H+L) polyclonal antibodies can be bought (Zymed Laboratories).

**[0023]** The substance comprising the part that recognizes human PD-1 and the part that recognizes the membrane protein on cell membrane of human PD-1-expressing cells means a substance that can simultaneously bind to an extracellular domain of human PD-1 and an extracellular domain of the membrane protein on cell membrane of the same cell. Concretely, they include bispecific antibodies comprising specific antibodies or fragments thereof to human PD-1 and specific antibodies or fragments thereof to the membrane protein on cell membrane of human PD-1-expressing cells.

**[0024]** The bispecific antibodies are unsymmetrical antibodies having two independent antigen-recognition sites with two different antigenic specificities. A well-known chemical method (Nisonoff, A., *et al.*, *Archives of Biochemistry and Biophysics*, 90;460-462 (1961), Brennan, M., *et al.*, *Science*, 299;81-83 (1985)) is already well-known as a preparation method of the bispecific antibodies. The bispecific antibodies can be obtained by respectively hydrolyzing two kinds of antibodies by enzyme and cutting disulfide bonds of the H chains by reducing agents, and then mixing and reoxidizing the different kind of antibodies. Recently, the preparation method using cross linking agents such as glutaraldehyde and carbodiimide has been disclosed (JP02-01556).

**[0025]** Technologies that directly produce the bispecific antibodies by using gene recombination technologies have been known. For example, the producing of bispecific antibodies (called single chain diabody) to carcinoembryonic antigen and *E. coli* beta-galactosidase has been reported in Alt, *FEBS Letter*, 454, 90 (1999). A heavy chain variable domain (VH) and other light chain variable domain (VL) of the fragment are connected with linkers to make them combine between two continuous domains on the same chain. A heavy chain variable domain (VH) and other light chain variable domain (VL) of the fragment are connected with linkers to make them combine between two continuous domains on the same chain. Therefore, VH and VL domain of the fragment are unavoidable to combine with complementary VL and VH domain of another fragment, as a result, two antigen binding sites are formed. Though 3-12 amino acid residues are preferable for the peptide linkers, the sequence is not limited (Hudson, P.J., *et al.*, *Journal of Immunology Medicine*, 231, 1-2; 177-189 (1999)).

**[0026]** As manufacturing of the bispecific antibodies using hybridomas, there is Reading, *et al.*'s method, that is, a method of producing hybrid hybridomas by further fusing two kinds of hybridomas that produce the monoclonal antibodies and then selecting hybrid hybridomas that produce target bispecific antibodies (US4474893).

**[0027]** The bispecific antibodies comprising specific antibodies or fragments thereof to human PD-1 and specific antibodies or fragments thereof to the membrane protein on cell membrane of human PD-1-expressing cells are antibodies that can simultaneously bind to the extracellular domain of human PD-1 and the extracellular domain of the membrane protein on cell membrane of the same cell.

**[0028]** The bispecific antibodies can be produced by the following method;

- (1) animals are sensitized by human PD-1 or human membrane protein as an immunogen,
- (2) splenic cells from the sensitized animals and myeloma cells from syngeneic animals are fused,
- (3) cells that produce monoclonal antibodies against the sensitizing antigen (human PD-1 or human membrane protein) are screened from the obtained hybridomas,
- (4) target antibodies-producing hybridomas are cloned,
- (5) the cloned antibodies-producing hybridomas are proliferated,
- (6) the produced antibodies are separated and refined,
- (7) the bispecific antibodies are produced by cross-linking the obtained anti-human PD-1 antibodies and anti-human membrane protein antibodies with linkers, or
- (8) they are further digested by pepsin and are separated, and refined to obtain  $F(ab')_2$ ,
- (9) each prepared  $F(ab')_2$  is reduced and is separated and refined,
- (10) the bispecific antibodies can be produced by cross-linking each prepared  $Fab_{SH}$  by linkers.

The linkers are not limited and may be anything that can link the part that recognizes the membrane protein of PD-1-expressing cells to the part that recognizes human PD-1, keeping an appropriate range. More concretely, they include peptides and amides etc.

The commercial products, for example, phenylenedimaleimide (Aldrich) can be used as the linkers.

When both or one of substances that respectively recognize the membrane protein of PD-1-expressing cells and human PD-1 is/are low-molecular weight organic compound(s),

- (11) using the antibodies produced by the above technique, the low-molecules that inhibit a bond between human membrane protein or human PD-1 and each antibody are found by measuring with a suitable detector,
- (12) the antibody can be produced by cross-linking the low-molecules, the antibodies, or the Fab with linkers.

[0029] It concretely explains each process as follows.

[0030] (1) In sensitization, it is preferable that human PD-1 or human membrane protein is administrated to peritoneal cavity or foot pat of sensitized animals. And, the sensitized animals have only to be animals such as mouse and rat etc. that the monoclonal antibody can be obtained in general and are not limited. For example, it is enough that 10-200

5  $\mu\text{g}$  of the antigen are administrated to mouse once.

[0031] A spleen of the sensitized animal that antibody titer has risen enough among the sensitized animals in previous (1) is removed. Suspension of the splenic cells is prepared according to the usual manner. Then, cell fusion of previous (2) is performed by adding polyethylene glycol (preferably PEG4000.) to the mixture with the obtained splenic cells and syngeneic myeloma cells at 37°C. Several kinds such as P3 $\times$ 63Ag8, P3/NS1/1-Ag4-1, and SP-2/0-Ag-14 are

10 known as mouse myeloma cells, and everything are obtained easily.

[0032] It is preferable that myeloma cells are HGPRT (hypoxanthine-guanine phosphoribosyltransferase)- cell lines that can not live in HAT media (a medium containing hypoxanthine, aminopterin, and thymidine), and don't secrete antibodies. SP-2/0-Ag-14 are preferably used.

[0033] Then, the obtained cell fusion mixtures are sown in 96 microwell plate at low density, which are cultured in HAT medium. By 1-2 weeks, unfused myeloma cells, hybridomas of myeloma cells, unfused splenic cells, and hybridomas of splenic cells die out, whereas only hybridomas of splenic cells and myeloma cells proliferate.

[0034] By reacting culture supernatant of hybridomas with solid phase antigens and measuring the antibodies that specifically adsorbs the antigens by using labeled second antibodies, screening of previous (3) judges whether hybridomas product the antibodies against human membrane protein or human PD-1 or not.

20 [0035] The process of (4) is performed by cloning antibody-producing hybridomas according to soft agar culture (*Monoclonal Antibodies*, 372 (1980)). In this case, it is also possible to use the limiting dilution.

[0036] In the process of (5), to obtain a large amount of antibody more efficiently, a method of administrating the hybridomas into mouse peritoneal cavity, and separating and refining them from peritoneal fluid can be used.

25 [0037] In the process of (6), usual methods such as salting out, ion-exchange chromatography, gel filtration, hydrophobic chromatography, and affinity chromatography etc. are used, and more effectively, affinity chromatography using protein A-sepharose CL-4B (Amersham Biosciences K.K.) can be used.

[0038] Since the bispecific antibodies of the present invention specifically bind, they can be used for purification and concentration such as affinity chromatography etc. of human PD-1.

30 [0039] In the process of (7), for example, cross linking agents can make sulfo-EMCS (N-(6-maleinimidecapoxy) succinimide sodium salt) bind to SH (mercapto) groups or amide groups of antibodies. First, either of antibodies is referred to amido coupling reactions with sulfo-EMCS. The unreacted sulfo-EMGS is separated by gel filtration. SH (mercapto) groups of the other antibody that have been reduced by 2-mercaptoethylamine etc. are referred to react with maleimide groups of sulfo-EMCS that have bound to the first antibody. The one that two kinds of antibodies were mutually cross-linked is separated by gel filtration.

35 [0040] In the process of (8), each antibody that be obtained in process (6) was digested by pepsin for 48 hours at 37°C. F(ab')<sub>2</sub> digested by pepsin are purified by usual methods such as salting out, ion-exchange chromatography, gel filtration, hydrophobic chromatography, and affinity chromatography etc. and more effectively, gel filtration using cefaclor S-200 (Amersham Biosciences K.K.) can be used.

40 [0041] In the process of (9), F(ab')<sub>2</sub> are reduced by 2-mercaptoethanol for 30 minutes at 30°C. The reduced Fab<sub>SH</sub> are purified by usual methods such as salting out, ion-exchange chromatography, gel filtration, hydrophobic chromatography, and affinity chromatography etc. and more effectively, gel filtration using cefaclor S-200 can be used.

[0042] In the process of (10), the other antibodies in Fab<sub>SH</sub> fraction are referred to bind with linkers. Cross linking agents have only to bind mercapto (SH) groups of Fab<sub>SH</sub>, which is referred to react with phenylenedimaleimide for 30 minutes at room temperature. Then, it is referred to react with 1.3 times more the other Fab<sub>SH</sub> for 4 hours at room

45 temperature. The obtained substance having bivalent specificity are purified by usual methods such as salting out, ion-exchange chromatography, gel filtration, hydrophobic chromatography, and affinity chromatography etc. and more effectively, gel filtration using cefaclor S-200 can be used.

[0043] In the process of (11), the antibodies obtained in process (6) can be used as they are, or as the one that are preferably labeled (for example, biotin-labeling and FITC-labeling etc.) by the common procedure. In ELISA, the antibodies are added to solid phase antigens. Then, when using enzyme-labeled secondary antibody and biotin-labeled antibody, the specific binding between the antibodies and the antigens can be measured by absorptiometer under the presence of chromophore substance, after adding enzyme-labeled streptavidin. Low-molecules that specifically recognize PD-1 or the membrane protein can be obtained by using this assay system.

50 [0044] In the process of (12), when the other is an antibody or Fab, it can bind to an antibody or Fab by introducing suitable functional groups to the obtained low-molecules. For example, if maleimide groups are introduced, it can bind to mercapto (SH) groups of the antibody or Fab. If both are low-molecules, molecules including both can be synthesized.

55 [0045] On the other hand, cDNA of each antibody can be separated from hybridomas that produce the monoclonal antibodies against each antigen. By transforming suitable host cells using expression vector containing DNA that both

cDNA or partial fragments thereof have been linked by gene recombination methods, the host cells can produce bispecific antibodies.

**[0046]** Concretely, the bispecific antibodies comprising the antibodies against human PD-1 or partial fragments thereof and the antibodies against the membrane protein on cell membrane of human PD-1-expressing cells or partial fragments thereof can be prepared by the following methods;

- (1) Each antibody gene is separated from each hybridoma that anti-human PD-1 monoclonal antibodies and anti-membrane protein monoclonal antibodies are respectively produced,
- (2) Variable region DNA of anti-human PD-1 monoclonal antibody gene and variable region DNA of anti-membrane protein monoclonal antibody gene are linked by using linker DNA. The expression vectors containing the linked DNA fragments are introduced to suitable host cells,
- (3) the cells are cultured under a suitable culture condition, and produced proteins are separated and refined.

**[0047]** Each process is concretely explained as follows.

**[0048]** The process of (1) comprises a process of separating RNA from hybridomas and a process of separating an antibody gene or cDNA encoding partial peptide thereof.

**[0049]** The process of separating the total RNA or mRNA from hybridomas can be performed according to a well-known method (a method described in Sambrook, J., *et al.*, *Molecular Cloning* (1989), Cold Spring Harbor Laboratory, or F. M. Ausubel., *et al.*, *Current Protocol in Molecular Biology*).

**[0050]** By using synthetic DNA primers having partial nucleotide sequences of the antibodies of the present invention, cDNA encoding the antibody genes of the present invention or partial peptide thereof can be amplified by Polymerase Chain Reaction (hereafter, called PCR). Or, by hybridization with probes labeled by using DNA fragments or the synthetic DNAs encoding a part or whole region of the antibodies of the present invention, the cDNA can be selected from cDNA contained in suitable vectors. The hybridization can be performed according to a well-known method. The antibody gene can be amplified by using the total RNA or mRNA by Reverse Transcriptase Polymerase Chain Reaction (Hereafter, called RT-PCR).

**[0051]** (2) As a method of producing the bispecific antibodies of the present invention,

(i) a method of synthesizing peptide and

(ii) a method of producing by gene recombination technologies *etc.* are enumerated, and the method of the described in (ii) is preferable, industrially.

**[0052]** The expression system (hosts-vector system) to produce peptide by using gene recombination technologies includes, for example, the expression system of bacillus, yeasts, insect cells, and mammalian cells.

**[0053]** The vector system includes *E. coli* plasmids (for example, pBR322, pBR325, pUC12, and pUC13), *Bacillus subtilis* plasmids (for example, pUB110, pTP5, and pC194), yeast plasmids (for example, pSH19 and pSH15), bacteriophages such as lambda phage, animal viruses such as retrovirus, vaccinia virus, and baculovirus, PA1-11, pXT1, pRc/CMV, pRc/RSV, and pcDNA1/Neo *etc.*

**[0054]** The promoter in the present invention has only to be an appropriate promoter corresponding to the gene expression host. For example, when animal cells are used as hosts, SR $\alpha$  promoter, SV40 promoter, LTR promoter, CMV promoter, and HSV-TK promoter *etc.* are enumerated. It is preferable to use CMV (cytomegalovirus) promoter, SR $\alpha$  promoter *etc.* In case of *Escherichia coli*, trp promoter, lac promoter, recA promoter,  $\lambda$ PL promoter, lpp promoter, and T7 promoter *etc.* are preferable. In case of *Bacillus* bacterium, SPO1 promoter, SPO2 promoter, and penP promoter *etc.* are preferable. In case of yeasts, PHO5 promoter, PGK promoter, GAP promoter, and ADH promoter *etc.* are preferable.

**[0055]** Additionally, the expression vector containing enhancer, splicing signal, polyA signal, selected marker, and SV40 duplication ori (hereafter, it may be called SV40ori.) *etc.* can be used, if necessary. The selected marker includes, for example, dihydrofolate reductase (hereafter, it may be called dhfr.) gene (Methotrexate (MTX) resistance), ampicillin resistance gene (hereafter, it may be called Amp<sup>r</sup>), and neomycin resistance gene (hereafter, it may be called Neo<sup>r</sup>. G418 resistance) *etc.* Especially, when dhfr gene is used as a selected marker using dhfr gene deficient chinese hamster cells, a target gene can be selected even by using the medium without thymidine. The signal sequence to suitable for the hosts can be added to N-terminal side of the protein of the present invention, if necessary. When the hosts are *Escherichia*, PhoA signal sequence and OmpA signal sequence *etc.* can be used. When the hosts are bacillus,  $\alpha$ -amylase signal sequence and subtilisin signal sequence *etc.* can be used. When the hosts are bacillus, MF $\alpha$  signal sequence, SUC2 signal sequence *etc.* can be used. When the hosts are animal cells,  $\alpha$ -insulin signal sequence,  $\alpha$ -interferon signal sequence, and antibody signal sequence *etc.* can be used, respectively. The transformants can be manufactured using the vector containing DNA encoding the constructed protein of the present invention.

**[0056]** By culturing *Escherichia* transformed with the expression vector in suitable medium, the target peptide is

obtained from the body cells. And, if a signal peptide (for example, a signal peptide of pelB) of bacteria is used, the target peptide is secreted in the periplasm. Further, it can be produced as fusion protein with other peptide. In case of the expression in mammalian cells, for example, by culturing mammalian cells transformed by suitable expression vectors including cDNA encoding a target protein in suitable medium, the target peptides are secreted into medium.

[0057] For example, *Escherichia*, *Bacillus* bacterium, yeasts, insect cells, insects, and animal cells *etc.* can be used as host cells. For example, *Escherichia coli* K12, DH1, JM103, JA221, HB101, C600, JM109, DH5, and DH5 $\alpha$  *etc.* can be used as concrete examples of *Escherichia*. For example, bacillus *Bacillus subtilis* MI114 *etc.* can be used as *Bacillus* bacterium. For example, *Saccharomyces cerevisiae* AH22, AH22R - NA87-11A, DKD-5D, or 20B-12, *Saccharomyces pombe* NCYC1913, or NCYC2036, and *Pichia pastoris* KM71 *etc.* can be used as yeasts. When virus is AcNPV, for example, *Spodoptera frugiperda* Cell (SF cell), MG1 cells derived from *Trichoplusia ni* midgut, High Five<sup>TM</sup> cells derived from *Trichoplusia ni* egg, cells derived from *Mamestra brassicae*, cells derived from *Estigmene acrea* *etc.* can be used as insect cells. When virus is BmNPV, cell lines derived from silkworm (*Bombyx mori* N cells; BmN cells) *etc.* can be used. For example, Sf9 cells (ATCC CRL1711) (Vaughn, J. L., *In Vivo*, 13; 213-217 (1977)) and Sf21 cells *etc.* can be used as Sf cells. For example, silkworm larvae *etc.* can be used as insects. For example, COS-1, COS-7, Vero, chinese hamster cell CHO (hereafter, it's abbreviated with CHO cells.), dhfr-deficient chinese hamster cell CHO (hereafter, it's abbreviated with CHO(dhfr-) cells), mouse L cells, mouse AtT-20, mouse myeloma cells, rat GH3, HEK293 T lymphocytes, and human FL cells *etc.* can be used as animal cells. For example, *Escherichia* transformation can be performed according to *Proc. Natl. Acad. Sci. (USA)*, 69, 2110 (1972). For example, *Bacillus* transformation can be performed according to *Molecular & General Genetic*, 168, 111 (1979). For example, yeasts transformation can be performed according to Becker, DM., *et al*, *Methods in Enzymology*, volume 194, p.182-187 (1991), or *Proc. Natl. Acad. Sci. (USA)*, 75, 1929 (1978). Insect cells or insects transformation can be performed according to *Bio Technology*, 6; 47-55 (1978). Animal cells transformation can be performed according to *CELL TECHNOLOGY SUPPLEMENT 8 NEW CELL TECHNOLOGY EXPERIMENTAL PROTOCOL*, Shujunsha, 263 (1995), or *Virology*, 52, 456 (1973).

[0058] (3) The obtained peptides are purified by usual methods such as salting out, ion-exchange chromatography, gel filtration, hydrophobic chromatography, and affinity chromatography *etc.*

[0059] In general, a substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO: 2, SEQ ID NO:4 or SEQ ID NO:11 is a polypeptide containing the amino acid sequence that is 90% or more, 95, 98, or 99% or more homologous to the polypeptide on producing.

[0060] A homologue of the substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:11 is a polypeptide containing the amino acid sequence that is at least 70% or more, preferably at least 80, 90, or 95% or more homologous to the polypeptide over a region of at least continuous 20 amino acids, preferably at least 30, 40, 60, or 100 amino acids.

[0061] A fragment of the substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:11 is a polypeptide containing at least 10 amino acids, preferably at least 15, 20, 25, 30, 40, 50, or 60 amino acids, and a homologue thereof is a polypeptide containing the amino acid sequence that is at least 70%, preferably at least 80, 90, or 95% or more homologous to the polypeptide over a region of at least continuous 10 amino acids, preferably at least continuous 15, 20, 25, 30, 40, 50, or 60 amino acids.

[0062] A substantially pure polypeptide comprising amino acid sequence that deleted one or several amino acids from a polypeptide comprising the amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:11 is a polypeptide comprising amino acid sequence that deleted one or two amino acids or more (preferably 1 to approx. 25, more preferably 1 to approx. 10, furthermore, preferably 1 to 5) from amino acid sequence.

[0063] A substantially pure polypeptide comprising amino acid sequence that has been substituted by one or several amino acids from a polypeptide comprising the amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:11 is a polypeptide comprising amino acid sequence that has been substituted by one or two amino acids or more (preferably 1 to approx. 25, more preferably 1 to approx. 10, furthermore, preferably 1 to 5).

[0064] A homologue of a polynucleotide comprising DNA the nucleotide sequence represented by SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:9 is a polynucleotide that is at least 70%, preferably at least 80, 90, or more preferably 95% or more homologous to the polynucleotide over a region of at least continuous 20, preferably at least continuous 30, 40, 60, or 100 nucleotide sequence.

[0065] A fragment of the polynucleotide comprising DNA the nucleotide sequence represented by SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:9 is a polynucleotide containing polynucleotide comprising at least continuous 20, preferably at least continuous 30, 40, 50, 60, or 100 nucleotide sequence.

[0066] A DNA hybridizing to DNA containing the nucleotide sequence represented by SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:9 under stringent condition includes, for example, approx. a DNA containing nucleotide sequence that is approx. 70% or more, preferably approx. 80% or more, more preferably approx. 90% or more, most preferably approx. 95% or more homologous to nucleotide sequence respectively represented by SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:9.

[0067] The hybridization can be performed according to a well-known method or, for example, a method described



in J. Sambrook, *Molecular Cloning*, 2nd, Coldspring Harbor Laboratory. When using a commercial library, it is possible to perform it according to an attached method described in directions for use. More preferably, it is possible to perform it under stringent condition. High-stringent condition is approx. 19-40 mM, preferably approx. 19-20 mM of NaCl concentration, at approx. 50-70°C, preferably approx. 60-65°C. Approx. 19 mM of sodium concentration at approx. 65°C is most preferable, especially.

Industrial applicability

Application to medicine:

**[0068]** The substance specific to human PD-1 of the present invention can be used for therapy and/or prevention for the following disease.

**[0069]** The substance specific to human PD-1 of the present invention is useful for therapy and/or prevention for neurodegenerative diseases (geriopsychosis, Alzheimer disease, Down syndrome, Parkinson's disease, Creutzfeldt-jakob disease, diabetic neuropathy, Parkinson syndrome, Huntington's disease, Machado-Joseph disease, amyotrophic lateral sclerosis, diabetic neuropathy, and Creutzfeldt Creutzfeldt-Jakob disease).

**[0070]** The substance specific to human PD-1 of the present invention is useful for therapy and/or prevention for diseases that accelerate the immune reaction, for example, autoimmune diseases (glomerular nephritis, arthritis, dilated cardiomyopathy-like disease, ulcerous colitis, Sjogren syndrome, Crohn disease, systemic erythematodes, chronic rheumatoid arthritis, multiple sclerosis, psoriasis, allergic contact dermatitis, polymyositis, pachyderma, periarteritis nodosa, rheumatic fever, vitiligo vulgaris, insulin dependent diabetes mellitus, Behcet disease, Hashimoto disease, Addison disease, dermatomyositis, myasthenia gravis, Reiter syndrome, Graves' disease, anaemia perniosa, Good-pasture syndrome, sterility disease, chronic active hepatitis, pemphigus, autoimmune thrombopenic purpura, and autoimmune hemolytic anemia).

**[0071]** The substance specific to human PD-1 of the present invention is useful for therapy and/or prevention for collagenosis (systemic lupus erythematosus, rheumatoid arthritis, systemic scleroderma, diffuse scleroderma, dermatomyositis, polymyositis, polymyositis, Sjogren's syndrome, mixed connective tissue disease, polyarteritis nodosa, and rheumatic fever *etc.*).

**[0072]** The substance specific to human PD-1 of the present invention is useful for therapy and/or prevention for organ graft rejection, allergic disease, and diseases caused by attenuation of immune reaction, which PD-1 participates, for example, tumour and infectious disease.

**[0073]** When using the substance specific to human PD-1 of the present invention for the above purpose, it is usually administered systemically or locally, and orally or parenterally.

**[0074]** The dosage is different depending on age, body weight, symptom, therapeutic effect, administration route, and duration of the treatment *etc.* For oral administration, generally, the dosage range from 0.1 mg to 100 mg per an adult is orally administered once to several times per day, or the dosage range from 0.01 mg to 30 mg per an adult is administered once to several times per day parenterally, suitably intravenously, and is intravenously administered for 1 to 24 hours per day continuously.

**[0075]** Since the dosage changes depending on various conditions as described above, there are cases in which doses lower or greater than the above dosage may be used.

**[0076]** When a composition of the present invention is administered, it is used as internal solid medicines and internal liquid medicines for internal use, and injections, external preparations, suppositories *etc.* for parenteral administration.

**[0077]** The internal solid medicines for oral administration include compressed tablets, pills, capsules, dispersing powders, granules *etc.* The capsules include hard capsules and soft capsules.

**[0078]** In case of such the solid medicines, one or more active compound(s) may be pharmaceutically manufactured as itself/themselves or a formulation with excipients (lactose, mannitol, glucose, microcrystal cellulose, and starch *etc.*), binders (hydroxypropylcellulose, polyvinyl pyrrolidone, and magnesium metasilicate aluminate *etc.*), disintegrators (cellulose calcium glycolate *etc.*), lubricants (magnesium stearate *etc.*), stabilizers, or solubilizers (glutamate and aspartic acid *etc.*) *etc.* according to usual methods. Further, they may be optionally coated by coatings (sucrose, gelatin, hydroxypropylcellulose, and hydroxypropylmethylcellulose phthalate *etc.*) or coated in the layer of two or more. Further, capsules of absorbable materials such as gelatin may be included.

**[0079]** The liquid compositions for oral administration include pharmaceutically acceptable waters, suspensions, emulsions, syrups, and elixirs *etc.* As to such liquid medicines, one or more active compound(s) may be dissolved, suspended, or emulsified to generally used diluent (purified water, ethanol or those mixing liquids *etc.*). Further, those liquid medicines may contain humectants, suspending agents, emulsifying agents, sweeteners, flavor agents, flavoring agents, preservatives, and buffers *etc.*

**[0080]** The injections for parenteral administration include solid injections that are dissolved or suspended to solution, suspension, emulsion, or time of use solvent. The injections are used by dissolving, levigating and melting one or more

activator(s) to the solvent. As the solvent, for example, water for injection, distilled saline, vegetable oil, propylene glycol, polyethylene glycol, alcohols such as ethanol *etc.* and these combinations are used. Further, this injection may include stabilizers, solubilizers (glutamate, aspartic acid, and polysorbate 80 (registered trademark) *etc.*), suspending agents, emulsifying agents, soothing agents, buffers, and preservatives *etc.* These are sterilized in the final process or are manufactured from the aseptic manipulation. Aseptic solid medicines may be manufactured as a freeze-drying product and may be used by making to be aseptic or dissolving to aseptic distilled water for injection or other solvents before use.

**[0081]** Other compositions containing one or more activator(s) for parenteral administration include liquid for external use, ointment drug, coating drug, inhalant, aerosol, suppository and pessary for intrarectal administration which is prescribed according to a routine procedure.

**[0082]** The sprays may contain a stabilizer such as sodium hydrogen sulfite besides the diluent generally used, a buffer giving isotonicity, and an isotonic medicine such as, for example, sodium chloride, sodium citrate, or citrates. Production methods of the sprays have been described in, for example, U.S. Patent 2,868,691 specification and U.S. Patent 3,095,355 specification in detail.

**[0083]** Since PD-1 relates to the immune reaction, PD-1 can also be used for screening *etc.*, the substance related to the immune reaction by measuring the expression of human PD-1 using the substance specific to human PD-1 of the present invention.

**[0084]** The substance specific to human PD-1 of the present invention comprises of a part that recognizes human PD-1, a part that recognizes the membrane protein on cell membrane of human PD-1-expressing cells, and a linker, and is an excellent substance that specifically recognizes human PD-1 and the membrane protein, and can transmit human PD-1 signal.

#### Brief Description of Drawings

**[0085]**

Figure 1 shows the construction of a bispecific antibody expression vector.

Figure 2 shows the reactivity of each bispecific antibody against X63 cell surface antigen expressed in CD3(-)/PD-1(+) cells and peripheral blood mononuclear cells (PBMC) surface antigen as CD3(+)/PD-1(-) cells. In this figure, filling areas of histogram represent control IgG and opening areas represent distribution of PD-1 or CD3 positive cell.

Figure 3 shows the effect on proliferative response of activated human peripheral blood T cells of bispecific antibody. In this figure, "-" represents Control IgG-adding group and "BsAb" represents the bispecific antibody-adding group, the spindle represents measurement absorbance, and \*\* represents  $P < 0.05$  in significance test.

#### Best Mode for Carrying out the Invention

**[0086]** The following examples explain the present invention more concretely, but don't limit the range of the present invention.

##### Example 1:

**[0087]** To obtain DNA encoding anti-human PD-1 antibody and anti-human CD3 antibody respectively, each total RNA was isolated from J110 (International Trust Number: FERM BP-8392) hybridoma and CD3 antibody hybridoma (obtained from ATCC : ATCC Number: CRL-8001). The operation was performed by using SV total Isolation System (trade name: purchase from Promega) according to the manufacturer instructions.

**[0088]** J110 hybridoma cDNA library and CD3 antibody hybridoma cDNA library were generated from total RNA (total RNA) by oligo dT prime method by using Ready-To-Go You-Prime First-Strand Beads (trade name: purchase from Amersham Pharmacia). The operation and procedure were done according to the manufacturer instructions.

**[0089]** cDNA of variable region of each IgG heavy chain and IgG light chain of anti-human PD-1 antibody and anti-human CD3 antibody was amplified by PCR reaction using Heavy Primers and Light Primers (the trade name: purchase from Amersham Pharmacia), respectively. PCR was carried out the following steps; as a first step at 95°C for 2 minutes and as cycle steps at 95°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for 40 seconds was repeated 30 times and was left at 72°C for 5 minutes as last step.

**[0090]** PCR product was separated by agarose gel electrophoresis. The expected size of DNA fragment was collected and was cloned into pGEM-T Easy Vector (trade name: purchase from Promega). Then, *E. coli* DH5 $\alpha$  was transformed with the plasmid. DNA of each IgG heavy chain (SEQ ID NO:1 or 5) and IgG light chain (SEQ ID NO:3 or 7) were sequenced to determine the consensus sequence.

## Example 2:

**[0091]** DNAs encoding bispecific antibodies were prepared by being respectively connected to the separated cDNAs in example 1. Fragment 1 was prepared by connecting IgG heavy chain cDNA (SEQ ID NO:1) of anti-human PD-1 antibody and IgG light chain cDNA (SEQ ID NO:7) of anti-human CD3 antibody by PCR using linker No.1 (SEQ ID NO:19), linker No.2 (SEQ ID NO:20), primer No.1 (SEQ ID NO:14), and primer No.2 (SEQ ID NO:15) (Figure 1). Fragment 2 was prepared by connecting IgG heavy chain cDNA (SEQ ID NO:7) of anti-human CD3 antibody and IgG light chain cDNA (SEQ ID NO:1) of anti-human PD-1 antibody by PCR using linker No.3 (SEQ ID NO:21), linker No.4 (SEQ ID NO:22), primer No.3 (SEQ ID NO:16), and primer No.4 (SEQ ID NO:17) (figure.1). Fragment 3 was prepared (Figure 1) by connecting fragment 1 and fragment 2 by PCR using linker No.5 (SEQ ID NO:23), linker No.6 (SEQ ID NO:24), primer No.5 (SEQ ID NO:18), and primer No.4 (SEQ ID NO:22) and then the nucleotide sequence was decided (SEQ ID NO:9).

**[0092]** Each PCR reaction was respectively performed. First PCR was performed by repeating 20 times the following steps; at 94°C for 30 seconds, at 40°C for 30 seconds, and at 72°C for 50 seconds. 2nd PCR was performed by repeating 30 times the following steps; at 94°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for 50 seconds, by using first PCR solution as a template.

## Primer No.2:

5'-TTTTTTAAGCTTACAGGTCCAGCTGCAGGAGTCA-3' (SEQ ID NO:14)

## Primer No.2:

5'-TTTTTTGCGGCCGCCCCGTTTATTTCCAACCTTTG-3' (SEQ ID NO:15)

## Primer No.3:

5'-TTTTTTAAGCTTACAGGTCCAGCTGCAGCAGTCT-3' (SEQ ID NO:16)

## Primer No.4:

5'-TTTTTTGCGGCCGCCCCGTTTGATTTCCAGCTTGG-3' (SEQ ID NO:17)

## Primer No.5:

5'-ATGAACTGGTACCAGCAGAAG-3'(SEQ ID NO:18)

## Linker No.1:

5'-AGGGACCACGGTCACCGTCTCCTCAGGTGGAGGCGGTTACAAAATTGTT  
CTCACCCAGTCTCCAG-3' (SEQ ID NO:19)

## Linker No.2:

5'-CTGGAGACTGGGTGAGAACAATTTGTGAACCGCCTCCACCTGAGGAGAC  
GGTGACCGTGGTCCCT-3' (SEQ ID NO:20)

Linker No.3:

5'-AGGCACCACTCTCACAGTCTCCTCAGGTGGAGGCGGTTTCAGACATCCAG  
ATGACCCAGTCTCCAG-3' (SEQ ID NO:21)

Linker No.4:

5'-CTGGAGACTGGGTCATCTGGATGTCTGAACCGCCTCCACCTGAGGAGAC  
TGTGAGAGTGGTGCCT-3' (SEQ ID NO:22)

Linker No.5:

5'-GGGGACAAAGTTGGAAATAAACCGGGGTGGAGGCGGTTTCAGGCGGAGGT  
GGCTCTGGCGGTGGCGGATCGCAGGTCCAGCTGCAGCAGTCTGGGG-3' (SEQ ID  
NO:23)

Linker No.6:

5'-CCCCAGACTGCTGCAGCTGGACCTGCGATCCGCCACCGCCAGAGCCACC  
TCCGCCTGAACCGCCTCCACCCGGTTTATTTCCAACCTTTGTCCCC-3' (SEQ ID  
NO:24)

[0093] DNA encoding the bispecific antibody prepared by the above method was cloned into expression vector pSecTag2/HygroA (trade name: purchase from Invitrogen). First, each of fragment 1 and fragment 3 was digested by restriction enzyme *HindIII*, *KpnI*, *KpnI*, and *NotI* and then was purified by agarose electrophoresis. Then, the DNA fragments were ligated in a *HindIII* and *NotI* digested pSecTag2/HygroA vector. *E. coli* DH5 $\alpha$  was transformed with the plasmid J110-CD3scDb-pSec/hygroA encoding the bispecific antibody was amplified, extracted, and purified (Figure 1).

Example 3:

[0094] Bispecific antibody protein was prepared by J110-CD3scDb-pSec/hygroA expression plasmid. The construct was transiently transfected into human kidney cell line 293T (ATCC Number: CRL-11268) with LipofectAMINE-plus (trade name: purchased from Invitrogen) and cultured for four days. The supernatant was sterilized with 0.22  $\mu$ m PVDF filter, and was concentrated using 40% PEG20000 solution. The concentrated supernatant was purified by HiTrap Cheating HP column (trade name: purchased from Amershampharmacia).

## Example 4:

**[0095]** The reactivity of the bispecific antibody (PD-1 and CD3) binding to cell surface antigens analyzed by FACScan.

**[0096]** 1 or 10  $\mu\text{g}$  of the bispecific antibodies were respectively added to human PD-1-expressed X63 cell lines as PD-1 positive/CD3 negative cells (CD3(-)/PD-1(+) cells) and human peripheral blood mononuclear cells as PD-1 negative/CD3 positive cells (CD3(+)/PD-1(-) cells), and incubated on ice. Immediately second antibodies were added and incubate on ice for 30 min. It was confirmed that the bispecific antibodies reacted to PD-1 and CD3.

## Example 5:

**[0097]** The activity of the bispecific antibody was evaluated as an effect on the proliferation of activated human peripheral blood T cells.

**[0098]** Concretely, PBMC were isolated from peripheral blood samples of healthy donors by Lymphoprep Tube (trade name: purchased from HYCOMED PHARMA). The operation and procedure were done according to the manufacturer instructions. Erythocytes were removed from PBMC by lysis with lysis buffer (0.8%  $\text{NH}_4\text{Cl}$ , 0.1%  $\text{KCO}_3$ , 1 mM EDTA). And purified T cell passed through Nylon Fiber ColumnT (trade name: purchased from Rosh) were resuspended to medium (RPM11640 medium including 10% fetal bovine serum).

**[0099]** Purified T cell ( $2 \times 10^6/\text{mL}/\text{well}$ ) were cultured for 60 hours with 1  $\mu\text{g}/\text{mL}$  anti-human CD28 antibody (the clone name: CD28.2 and purchased from Pharmingen) in 24 well-plate that has were pre-coated with 5  $\mu\text{g}/\text{mL}$  anti-human  $\alpha\beta\text{TCR}$  antibody (the clone name: T10B9.1A-31 and purchased from Pharmingen). Activated T cells were rested for 12 hours, and resting T cells ( $1 \times 10^6/100 \mu\text{L}/\text{well}$ ) were restimulated adding 1  $\mu\text{g}/\text{well}$  of bispecific antibody in 96 well-plate that were pre-coated with 0.1  $\mu\text{g}/\text{mL}$  anti- $\alpha\beta\text{TCR}$  antibody. 48 hours later, T cell proliferation was determined=by BrdU incorporation using Cell Proliferation ELISA (the trade name: purchased from Rosh). Figure 3 shows the result.

**[0100]** The bispecific antibodies have significantly decreased the proliferation of the activated human peripheral blood T cells.

## SEQUENCE LISTING

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HONJO, Tasuku

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 Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe  
                                  50                      55                      60  
 35 Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr  
 65                      70                      75                      80  
 Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys  
                                  85                      90                      95  
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 45 <213> Mus musculus

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 50 ttcaggggca gtgggtcttg gacctttac tctctcaca tcagcggcat ggaggctgaa 240  
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 acaagattgg aaataaacg g 321

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 <211> 107  
 55 <212> PRT  
 <213> Mus musculus

<400> 8  
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 35 40 45  
 Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser  
 50 55 60  
 Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu  
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 <212> DNA  
 <213> Artificial Sequence  
 <223> Polynucleotide sequence coding bispecific antibody

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 ctagtgtcca gccatctgtt gtttgccctt ccccggtgct ttccttgacc ctggaagggt 1920  
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 1982

<210> 10  
 <211> 1982  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (207)..(1823)  
 <223> Polynucleotide and polypeptide sequence coding bispecific antibody



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	ctctggctaa	ctagagaacc	cactgcttac	tggttatcg	aaattaatac	gactcactat									180
5	agggagaccc	aagctggcta	gccacc atg	gag aca	gac aca	ctc ctg	cta	tgg							233
			Met	Glu Thr	Asp Thr	Leu Leu	Leu Trp								
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	gta ctg	ctg ctc	tgg gtt	cca ggt	tcc act	ggt gac	gcg gcc	cag ccg							281
	Val Leu	Leu Leu	Trp Val	Pro Gly	Ser Thr	Gly Asp	Ala Ala	Gln Pro							
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	Ala Arg	Arg Ala	Val Arg	Ser Ser	Leu Gln	Val Gln	Leu Gln	Glu Ser	Gly						
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	cct ggc	ctg gtg	aaa cct	tct cag	tct ctg	tcc ctc	acc tgc	act gtc							377
	Pro Gly	Leu Val	Lys Pro	Ser Ser	Gln Ser	Leu Ser	Leu Thr	Cys Thr	Val						
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15	act ggc	cac tca	atc acc	agt gat	tat ggc	tgg aac	tgg atc	cgg cag							425
	Thr Gly	His Ser	Ile Thr	Ser Ser	Asp Tyr	Ala Trp	Asn Trp	Ile Arg	Gln						
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	ttt cca	gga gac	aaa ctg	gag tgg	atg ggc	tac ata	agc tac	agt ggt							473
	Phe Pro	Gly Asp	Lys Leu	Glu Trp	Met Gly	Tyr Ile	Ser Tyr	Ser Gly							
		75		80		85									
20	tac act	acc tac	aac cca	tct ctg	aaa agt	cga gtc	tct atc	act cga							521
	Tyr Thr	Thr Tyr	Asn Pro	Ser Ser	Leu Lys	Ser Arg	Val Ser	Ile Thr	Arg						
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	gac aca	tcc aag	aac cag	ttc ttc	ctg cag	ttg aat	tct gtg	act act							569
	Asp Thr	Ser Lys	Asn Gln	Phe Phe	Leu Gln	Leu Asn	Ser Val	Thr Thr							
			110			115		120							
25	gag gac	aca gcc	aca tac	ttc tgt	gca aga	gac ctt	gat tac	ggc ccc							617
	Glu Asp	Thr Ala	Thr Tyr	Phe Cys	Ala Arg	Asp Leu	Asp Tyr	Gly Pro							
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	Trp Phe	Ala Tyr	Trp Gly	Gln Gln	Gly Thr	Thr Val	Thr Ser	Ser Gly							
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	Gly Gly	Gly Ser	Gln Ile	Val Leu	Thr Gln	Ser Pro	Ala Ile	Met Ser							
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	gca tct	cca ggg	gag aag	gtc acc	atg acc	tgc agt	gcc agc	tca agt							761
	Ala Ser	Pro Gly	Glu Lys	Val Thr	Met Thr	Cys Ser	Ala Ser	Ser Ser							
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	Val Ser	Tyr Met	Asn Trp	Tyr Gln	Gln Ser	Gly Thr	Ser Ser	Pro Lys							
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	aga tgg	att tat	gac aca	tcc aaa	ctg gct	tct gga	gtc cct	gct cac							857
	Arg Trp	Ile Tyr	Asp Thr	Ser Lys	Leu Ala	Ser Gly	Val Pro	Ala His							
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	Met Glu	Ala Glu	Asp Ala	Ala Thr	Tyr Tyr	Cys Gln	Gln Trp	Ser Ser							
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	Asn Pro	Phe Thr	Phe Gly	Ser Ser	Gly Thr	Lys Leu	Glu Ile	Asn Arg	Gly						
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	Gly Gly	Gly Ser	Gly Gly	Gly Gly	Ser Ser	Gly Gly	Gly Ser	Gln Val							
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50	cag ctg	cag cag	tct ggg	gct gaa	ctg gca	aga cct	ggg gcc	tca gtc							1097
	Gln Leu	Gln Gln	Ser Gly	Ala Glu	Leu Ala	Arg Pro	Gly Ala	Ser Val							
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	aag atg	tcc tgc	aag gct	tct ggc	tac acc	ttt act	agg tac	acg atg							1145
	Lys Met	Ser Cys	Lys Ala	Ser Gly	Tyr Thr	Phe Thr	Arg Tyr	Thr Met							
			300			305		310							
55	cac tgg	gta aaa	cag agg	cct gga	cag ggt	ctg gaa	tgg att	gga tac							1193
	His Trp	Val Lys	Gln Arg	Pro Gly	Gln Gly	Leu Glu	Trp Ile	Gly Tyr							
			315			320		325							

	att	aat	cct	agc	cgt	ggt	tat	act	aat	tac	aat	cag	aag	ttc	aag	gac	1241
	Ile	Asn	Pro	Ser	Arg	Gly	Tyr	Thr	Asn	Tyr	Asn	Gln	Lys	Phe	Lys	Asp	
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5	aag	gcc	aca	ttg	act	aca	gac	aaa	tcc	tcc	agc	aca	gcc	tac	atg	caa	1289
	Lys	Ala	Thr	Leu	Thr	Thr	Asp	Lys	Ser	Ser	Ser	Thr	Ala	Tyr	Met	Gln	
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	Leu	Ser	Ser	Leu	Thr	Ser	Glu	Asp	Ser	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	
					365				370					375			
10	tat	tat	gat	gat	cat	tac	tgc	ctt	gac	tac	tgg	ggc	caa	ggc	acc	act	1385
	Tyr	Tyr	Asp	Asp	His	Tyr	Cys	Leu	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Thr	
			380				385						390				
	ctc	aca	gtc	tcc	tca	ggt	gga	ggc	ggt	tca	gac	atc	cag	atg	acc	cag	1433
	Leu	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Asp	Ile	Gln	Met	Thr	Gln	
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15	tct	cca	gcc	tcc	cta	tct	gca	tct	gtg	gga	gaa	act	gtc	acc	ctc	aca	1481
	Ser	Pro	Ala	Ser	Leu	Ser	Ala	Ser	Val	Gly	Glu	Thr	Val	Thr	Leu	Thr	
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	Cys	Arg	Ala	Ser	Glu	Asn	Ile	His	Asn	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	
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	Lys	Gln	Gly	Lys	Ser	Pro	Gln	Leu	Leu	Val	Tyr	Asn	Val	Lys	Thr	Leu	
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	Ala	Asp	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Gln	
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25	tat	tct	ctc	aag	atc	aac	agc	ctg	cag	cct	gaa	gat	ttt	ggg	agt	tat	1673
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	tac	tgt	caa	cat	ttt	tgg	agt	agt	ccg	tgg	acg	ttc	ggt	gga	ggc	acc	1721
	Tyr	Cys	Gln	His	Phe	Trp	Ser	Ser	Pro	Trp	Thr	Phe	Gly	Gly	Gly	Thr	
	490					495				500						505	
30	aag	ctg	gaa	atc	aaa	cgg	gcg	gcc	gct	cga	gga	ggg	ccc	gaa	caa	aaa	1769
	Lys	Leu	Glu	Ile	Lys	Arg	Ala	Ala	Ala	Arg	Gly	Gly	Pro	Glu	Gln	Lys	
					510					515					520		
	ctc	atc	tca	gaa	gag	gat	ctg	aat	agc	gcc	gtc	gac	cat	cat	cat	cat	1817
	Leu	Ile	Ser	Glu	Asp	Leu	Asn	Ser	Ala	Val	Asp	His	His	His	His	His	
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	His	His															
35	atctgttgtt	tgccccctccc	ccgtgccttc	cttgaccctg	gaaggtgccca	ctcccactgt											1933
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				100					105					110			
55	Phe	Leu	Gln	Leu	Asn	Ser	Val	Thr	Thr	Glu	Asp	Thr	Ala	Thr	Tyr	Phe	
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 5 Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met Asn Trp Tyr  
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 Gln Gln Lys Ser Gly Thr Ser Pro Lys Arg Trp Ile Tyr Asp Thr Ser  
 195 200 205  
 Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser Gly Ser Gly  
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 10 Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu Asp Ala Ala  
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 25 Asp Ser Ala Val Tyr Tyr Cys Ala Arg Tyr Tyr Asp Asp His Tyr Cys  
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 385 390 395 400  
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 405 410 415  
 30 Ser Val Gly Glu Thr Val Thr Leu Thr Cys Arg Ala Ser Glu Asn Ile  
 420 425 430  
 His Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Gln Gly Lys Ser Pro Gln  
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 465 470 475 480  
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 485 490 495  
 Ser Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala  
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&lt;210&gt; 13

&lt;211&gt; 1434

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

20 &lt;223&gt; Polynucleotide sequence coding bispecific antibody

&lt;400&gt; 13

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&lt;213&gt; Artificial Sequence

45 &lt;223&gt; PCR primer oligonucleotide

&lt;400&gt; 14

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&lt;210&gt; 15

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#### 40 Claims

1. A substance specific to human PD-1 comprising a part that recognizes human PD-1, a part that recognizes a membrane protein on cell membrane of human PD-1-expressing cells, and a linker.
- 45 2. The substance specific to human PD-1 according to claim 1, wherein the part that recognizes human PD-1 is an antibody against human PD-1 or a partial fragment thereof.
3. The substance specific to human PD-1 according to claim 1, wherein the part that recognizes a membrane protein on cell membrane of human PD-1-expressing cells is an antibody against the membrane protein or a partial fragment thereof.
- 50 4. The substance specific to human PD-1 according to claim 1, comprising an antibody against human PD-1 or a partial fragment thereof, an antibody against the membrane protein on cell membrane of human PD-1-expressing cells or a partial fragment thereof, and a linker.
- 55 5. The substance specific to human PD-1 according to any one of claims 1, 3 and 4, wherein the membrane protein is human T cell receptor complex or human B cell receptor complex.

6. The substance specific to human PD-1 according to claim 1 or 4, wherein the linker is a peptide.
7. A bispecific antibody comprising an antibody against human PD-1 or a partial fragment thereof, an antibody against human T cell receptor complex or a partial fragment thereof, and a linker.
8. A bispecific antibody comprising an antibody against human PD-1 or a partial fragment thereof, an antibody against human B cell receptor complex or a partial fragment thereof, and a linker.
9. The bispecific antibody according to claim 7 or 8, wherein the linker is a peptide.
10. A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:2, a homolog thereof, a fragment thereof or a homolog of the fragment, or a polypeptide comprising the amino acid sequence to which 1-10 amino acid(s) of the polypeptide is/are deleted, substituted, and/or added, wherein the polypeptide composes an antibody against human PD-1.
11. A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:4, a homolog thereof, a fragment thereof or a homolog of the fragment, or a polypeptide comprising the amino acid sequence to which 1-10 amino acid(s) of the polypeptide is/are deleted, substituted, and/or added, wherein the polypeptide composes an antibody against human PD-1.
12. A polypeptide complex comprising the polypeptides according to claims 10 and 11.
13. A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:11, a homolog thereof, a fragment thereof or a homolog of the fragment, or a polypeptide comprising the amino acid sequence to which 1-10 amino acid(s) of the polypeptide is/are deleted, substituted, and/or added.
14. A substantially pure polypeptide comprising the amino acid sequence represented by SEQ ID NO:11, a homolog thereof, a fragment thereof or a homolog of the fragment, or a polypeptide comprising the amino acid sequence to which 1-10 amino acid(s) of the polypeptide is/are deleted, substituted, and/or added, which is the bispecific antibody according to any one of claims 7 to 9.
15. A polynucleotide encoding the polypeptide according to any one of claims 10, 11, 13 and 14, a homolog thereof or a complementary polynucleotide thereof, or a fragment thereof or a homolog of the fragment.
16. A polynucleotide comprising the nucleotide sequence represented by SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:9, a homolog thereof or a complementary polynucleotide thereof, or a fragment thereof or a homolog of the fragment.
17. A duplication or expression vector comprising the polynucleotide according to claim 15 or 16.
18. A host cell transformed by the duplication or expression vector according to claim 17.
19. A manufacturing method of the substance according to any one of claims 1 to 6, comprising culturing the host cell according to claim 18 under conditions to express the substance.
20. A manufacturing method of the bispecific antibody according to any one of claims 7 to 9, comprising culturing the host cell according to claim 18 under conditions to express the bispecific antibody.
21. A manufacturing method of the polypeptide according to any one of claims 10 to 14, comprising culturing the host cell according to claim 18 under conditions to express the polypeptide.
22. A therapeutic and/or preventive pharmaceutical composition for human PD-1 related-diseases, comprising an effective amount of the substance according to any one of claims 1 to 6, the bispecific antibody according to any one of claims 7 to 9, the polypeptide complex according to claim 12, or the polypeptide according to claim 13 or 14.
23. The therapeutic and/or preventive pharmaceutical composition according to claim 22, wherein the human PD-1 related-diseases are diseases selected from neurodegenerative disease, autoimmune disease, collagenosis, organ transplantation rejection, tumour, and infectious disease.

- 4 24. The therapeutic and/or preventive pharmaceutical composition according to claim 22, wherein the neurodegenerative diseases are diseases selected from geropsychosis, Alzheimer disease, Down syndrome, Parkinson's disease, Creutzfeldt-jakob disease, diabetic neuropathy, Parkinson syndrome, Huntington's disease, Machado-Joseph disease, amyotrophic lateral sclerosis, diabetic neuropathy, and Creutzfeldt Creutzfeldt-Jakob disease.
- 5
25. The therapeutic and/or preventive pharmaceutical composition according to claim 22, wherein the autoimmune diseases are diseases selected from glomerular nephritis, arthritis, dilated cardiomyopathy-like disease, ulcerous colitis, Sjogren syndrome, Crohn disease, systemic erythematodes, chronic rheumatoid arthritis, multiple sclerosis, psoriasis, allergic contact dermatitis, polymyositis, pachyderma, periarteritis nodosa, rheumatic fever, vitiligo vulgaris, insulin dependent diabetes mellitus, Behcet disease, Hashimoto disease, Addison disease, dermatomyositis, myasthenia gravis, Reiter syndrome, Graves' disease, anaemia perniciousa, Goodpasture syndrome, sterility disease, chronic active hepatitis, pemphigus, autoimmune thrombopenic purpura, and autoimmune hemolytic anemia.
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- 45
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- 55



FIG. 1

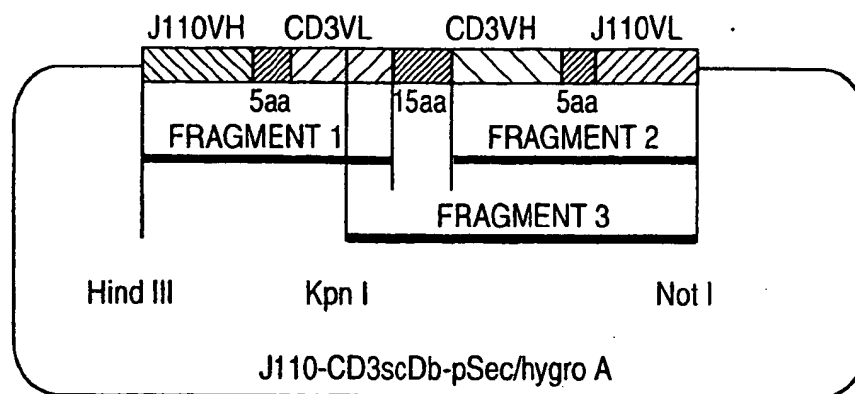


FIG. 2

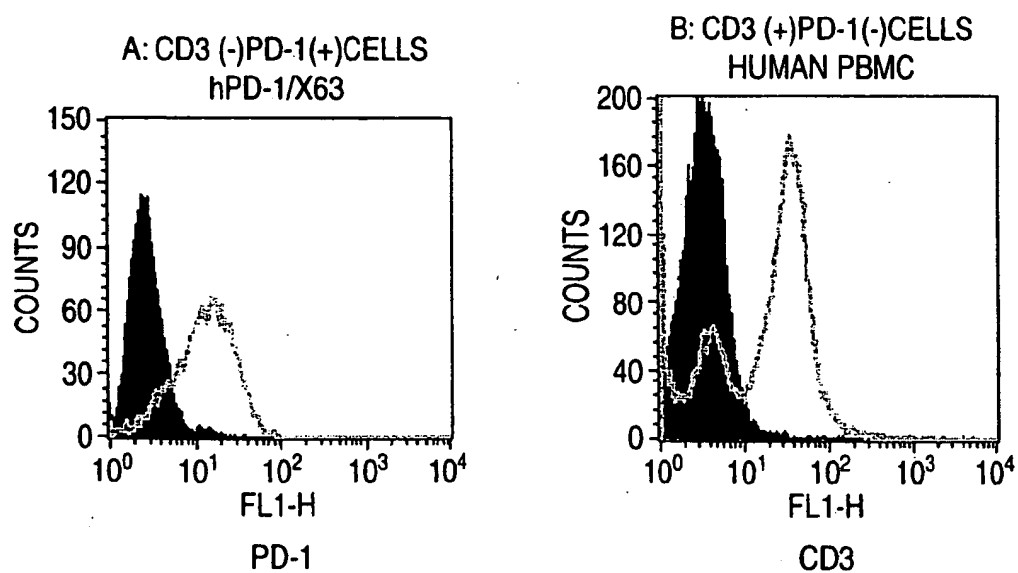
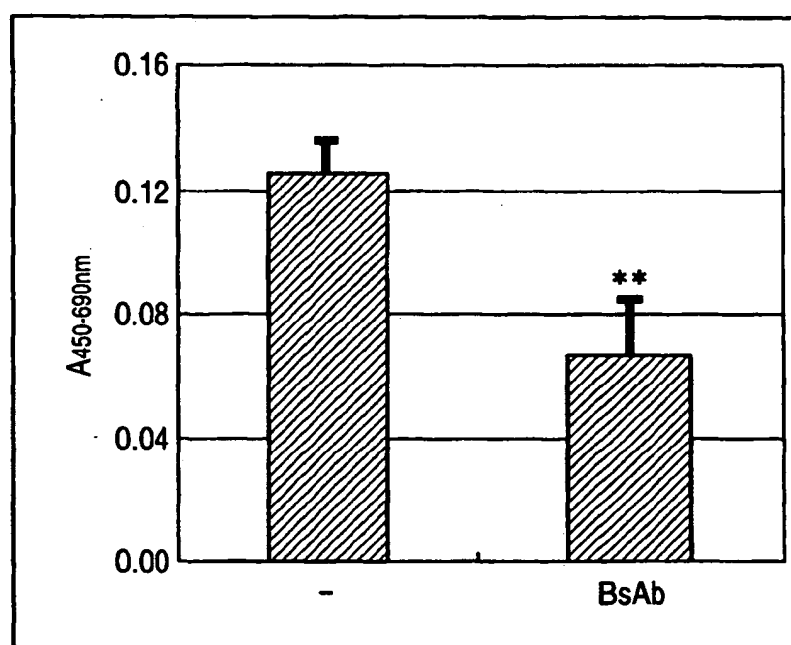


FIG. 3



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2004/000549

## A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl<sup>7</sup> C12N15/13, C07K16/46, C12P21/08, C12N5/10, A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl<sup>7</sup> C12N15/00-15/90, C07K14/00-16/46, C12P1/00-41/00,  
C12N1/00-9/99, A61K31/00-48/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

SwissProt/PIR/GeneSeq, GenBank/EMBL/DDBJ/GeneSeq, WPI (STN),  
BIOSIS (STN), MEDLINE (STN)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Wayne W. Hancock <sup>3</sup> et al., "Programmed Death-1 Targeting Can Promote Allograft Survival.", The Journal of Immunology, Vol.169, (2002), pages 6546 to 6553	1-25
Y	Honjo T. et al., "PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2.", Eur.J.Immunol., Vol.32, No.3, (2002), pages 634 to 643	1-25
Y	Margitta Alt et al., "Novel tetravalent and bispecific IgG-like antibody molecules combining single-chain diabodies with the immunoglobulin 1 Fc or CH3 region.", FEBS Letters, Vol.454, Issues 1-2, (1999), pages 90 to 94	1-25

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search  
02 June, 2004 (02.06.04)Date of mailing of the international search report  
22 June, 2004 (22.06.04)Name and mailing address of the ISA/  
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

Form PCT/ISA/210 (second sheet) (January 2004)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2004/000549

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Peter J. et al., "High avidity scFv multimers; diabodies and triabodies.", Journal of Immunological Methods, Vol.231, (1999), pages 177 to 189	1-25
Y	WO 2001/064249 A1 (Chugai Pharmaceutical Co., Ltd.), 07 September, 2001 (07.09.01), & JP 2001-563146 A	1-25

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2004/000549

**Box No. I** Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material

☒ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☐ in written format

☒ in computer readable form

c. time of filing/furnishing

☐ contained in the international application as filed

☒ filed together with the international application in computer readable form

☐ furnished subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: